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ORIGINAL INVESTIGATION

Significant association of *DRD1* with nicotine dependence

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Abstract Epidemiologic studies have strongly implicated genetics in smoking behavior. Genes in the dopaminergic system, which mediates the reinforcing and dependence-producing properties of nicotine, are plausible candidates for roles in nicotine dependence (ND). In this study, we examined five single-nucleotide polymorphisms (SNPs) within or near the dopamine D_1 receptor gene (*DRD1*) for their association with ND, which was assessed by smoking quantity (SQ), the Heaviness of Smoking Index (HSI), and the Fagerström Test for ND (FTND). The samples were obtained from 2,037 participants representing 200 European American (EA) and 402 African American (AA) families. Although we found significant associations of SNPs rs265973, rs686, and rs4532 in the AA sample; of rs4532 in the EA sample; and of rs265975, rs686, and rs4532 in the pooled sample with various ND measures, only the association of rs686 in the AA sample and of rs686 and rs4532 in the pooled sample remained significant after correction for multiple testing. Haplotype-based association analysis revealed that haplotype C-T-A, formed by rs265973, rs265975, and rs686, was significantly associated with all three ND measures in both the AA and the pooled sample. Another haplotype, T-A-T, formed by rs265975, rs686, and rs4532, showed a significant association with FTND in the pooled sample. Furthermore, in a luciferase reporter assay, rs686, located in the 3′ untranslated region, caused differential luciferase activities, indicating that rs686 is a functional polymorphism affecting expression of *DRD1*.

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Introduction

Nicotine dependence (ND) is highly prevalent and destructive throughout the world. According to the World Health Organization (WHO 2005), approximately 1.3 billion people worldwide smoke tobacco or related products, and tobacco smoking is responsible for the deaths of approximately 5 million people annually, which makes tobacco use a serious international health problem. Like other forms of substance dependence, ND has been demonstrated to be heritable, with an estimated heritability of at least 50% in individual differences in liability to ND (Li et al. 2003; Sullivan and Kendler 1999). Thus, identification of susceptibility genes for ND will provide insight into the etiology of nicotine addiction, and deeper understanding of the genetic risk factors for ND will be of great social and economic value. Considerable effort has been expended through linkage and association approaches to elucidate the specific genes involved in ND (Ho and Tyndale 2007; Li 2006; Li et al. 2004).

Nicotine is the primary addictive component of tobacco smoke that stimulates release of the neurotransmitter dopamine from neurons in the ventral tegmental area, an action

thought to underlie smoking's rewarding effects (Nisell et al. 1994; Pontieri et al. 1996). The dopaminergic pathway in the brain plays a prominent role in the pathogenesis of ND as well as in addiction to other drugs. In consequence, a great deal of attention has been devoted to determining whether variation in genes with dopaminergic function could account for the heritable variation in susceptibility to ND (Ho and Tyndale 2007; Li 2006; Li et al. 2004). As dopamine receptors mediate the effects of dopamine, they are candidates for genetic study of ND. Five dopamine receptors have been identified and characterized in human and have been classified into two groups: D_1-like (D_1 and D_5) and D_2-like (D_2, D_3, and D_4). In this study, we focused on *DRD1* to determine whether it is associated with ND.

Gene *DRD1* is located at chromosome 5q35.1 and contains two exons separated by a small intron in the 5′ untranslated region (UTR). In genome-wide linkage scans, a region near *DRD1* showed significant linkage to cigarette consumption and smoking initiation (Duggirala et al. 1999; Saccone et al. 2003; Vink et al. 2006), and *DRD1* has been suggested as a potential target for ND by both Duggirala et al. (1999) and Vink et al. (2006). However, so far, only one study found an association of the *Dde*I polymorphism (called rs4532 in the NCBI dbSNP database and this study) in *DRD1* with current smoking (Comings et al. 1997). The *Dde*I polymorphism is an A→G transition in the 5′ UTR (A-48G), one of four polymorphisms initially identified by single-strand conformational analysis of *DRD1* (Cichon et al. 1994). The other three are *Bst*N1 (G-94A) in the 5′ UTR, rare and synonymous *Pvu*I (G1263A) for codon 421, and *Bsp*1286I (T1403C) in the 3′ UTR (called rs686 in the NCBI dbSNP database and this study). Although several other polymorphisms around *DRD1* were identified later, none resulted in a change of an amino acid residue. Cichon et al. (1996) screened the 5′ flanking regulatory region of *DRD1* but found no polymorphism to have an important influence on the transcriptional activity.

In addition to ND, polymorphisms within or near *DRD1*, especially the *Dde*I polymorphism (rs4532), have been studied widely for genetic association with other neuropsychiatric diseases (Wong et al. 2000). Although most of them failed to show association, some have been revealed to be significantly associated with bipolar disorder (Del Zompo et al. 2007; Severino et al. 2005), attention deficit hyperactivity disorder (ADHD) (Bobb et al. 2005; Misener et al. 2004), and alcoholism (Kim et al. 2007; Limosin et al. 2003). As yet, little evidence for functional polymorphisms of *DRD1* has been reported.

To investigate whether *DRD1* is associated with ND, we examined five single-nucleotide polymorphisms (SNPs) within or near *DRD1* in our family-based association study. We not only provide evidence for significant association of *DRD1* with ND but also reveal that rs686 represents a causative polymorphism.

Materials and methods

Subjects

We recruited the participants in this study, who were of either African American (AA) or European American (EA) origin, primarily from the Mid-South states in the USA from 1999 to 2004, a group we refer to as the "Mid-South Tobacco Family (MSTF)" cohort (Beuten et al. 2006, 2007; Li et al. 2005, 2007). The cohort includes 2,037 subjects in 602 nuclear families, with 671 subjects in 200 EA families and 1,366 subjects in 402 AA families. The detailed demographic and clinical characteristics of the participants have been described (Beuten et al. 2006, 2007; Li et al. 2005, 2007). All participants provided informed consent. The study protocol and forms/procedures were approved by all participating Institutional Review Boards.

For each smoker, the degree of ND was ascertained by three commonly used measures: Smoking Quantity (SQ; defined as the number of cigarettes smoked per day), the Heaviness of Smoking Index (HSI; 0-6 scale), and the Fagerström Test for ND (FTND; 0–10 scale) (Heatherton et al. 1991). All three measures have been used consistently in our previously reported genetic studies on ND (Beuten et al. 2007; Beuten et al. 2006; Li et al. 2005; Li et al. 2007).

DNA extraction, SNP selection, and genotyping

Each genomic DNA sample was extracted from peripheral blood using the Maxi blood DNA extraction kit from Qiagen (Valencia, CA, USA). Five SNPs were selected from the NCBI dbSNP database to cover the whole region of *DRD1*. Published reports on *DRD1* polymorphisms, minor allele frequency, functional potential, and validation were considered in SNP selection. Information on these SNPs, including their IDs, allelic variants, contig positions, heterozygosities, and site functions, was obtained from the NCBI dbSNP database (Table 1). The *Taq*Man primer/probe set designed for each SNP allele was purchased from Applied Biosystems (Foster City, CA, USA), and their sequences are also provided in Table 1. The PCRs for genotyping were performed in a 384-well microplate format with *Taq*Man universal PCR master mix and a standard amplification protocol as routinely used by our laboratory (Beuten et al. 2006, 2007; Li et al. 2005, 2007). The following allelic discrimination analysis was carried out using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems).

Table 1 SNP characteristics and sequences of *Taq*Man probes designed for allele detection

SNP no.	dbSNP ID	Allele	Heterozygosity	Contig position (NT_023133)	SNP location	Sequences for allele detection (5′−3′)
1	rs265973	C/T	0.493	19670285	3′ near gene	F: GACCCTAGGAGCAAGCAATGTTATTA R: GGCAATAAGGTGAGGGTTCTAGTTT P: CTGGTCATAG/ATACCTTC
2	rs265975	C/T	0.493	19671781	3′ near gene	F: AGTAAGCCATCTCCCTGTTTTGTT R: GGGTAGAGTGTAAGAAAACGTTTGAGA P: AAAAACACGG/ACCTTGTG
3	rs686	A/G	0.453	19678286	Exon 2 (3′ UTR)	F: TCAGAGTCTCACCGTACCTTAGTTT R: CCCAAAAGCTAGAGGAGATTGCT P: CTTAATAGCAAA/GCCCC
4	rs4532	C/T	0.370	19679736	Exon 1 (5′ UTR)	F: AGCAATCTGGCTGTGCAAAGT R: CATCTTCCTAAGAGAAAGCACATCAG P: CCTGCTTG/AGGAACT
5	rs2168631	A/G	0.428	19685588	5′ near gene	F: GTGTTTTCTATTGTTGCACCTGCTC R: CTACTCAGTTAAGCCTATAAAAGGCAGAA P: TTCGTTCCA/GTTTTTC

All SNP information is obtained from NCBI SNP database

Individual SNP- and haplotype-based association analysis

The PedCheck program (O'Connell and Weeks 1999) was used to identify any inconsistent Mendelian inheritance, nonpaternity, or genotyping errors. Among over than 10,000 assays, 22 inconsistencies (12 in the AA samples, 0.18%; and 10 in the EA samples, 0.3%) were detected and were excluded from the following association analyses. To verify data quality, we also checked the genotyping results for any significant departure from Hardy–Weinberg equilibrium (HWE). Individual SNP association was determined by the Pedigree-Based Association Test (PBAT) program with generalized estimating equations (Lange et al. 2004). Pair-wise linkage disequilibrium (LD) between all SNP markers was evaluated by the Haploview program (Barrett et al. 2005) with the option of determining haplotype blocks according to the criteria defined by Gabriel et al. (2002). Haplotype-based association was identified by the Family-Based Association Test (FBAT) program with the option of computing *P* values of the *Z* statistic using Monte Carlo sampling (Horvath et al. 2004). The AA and EA samples were analyzed separately, with sex and age included as covariates in both PBAT and FBAT analysis. Additional ethnicity covariate was employed in analysis of the pooled sample. All three ND measures were analyzed under the additive model. All associations found to be significant were corrected for multiple testing according to the SNP spectral decomposition (SNPSpD) approach (Nyholt 2004) for individual SNP analysis, and using Bonferroni correction by dividing the significance level by the number of major haplotypes (frequency >5.0%) for haplotype-based association analysis.

Vector construct, cell transfection, and luciferase assay

The 3′ UTR of *DRD1* was PCR amplified from a human genomic DNA with the following primers: 5′-acgttctagaa ctcgcagatgaatcctg-3′ (forward) and 5′-acgttctagaagttacacat gaacatttag-3′ (reverse), on the basis of a cDNA sequence of X58987 from GenBank. The fragment of 726 basepairs was cloned into the *Xba*I site of the pGL3-Promoter vector (Promega, Madison, WI, USA; designated pLuc hereafter) and was sequenced to be a T-allele-containing 3′ UTR (pLuc-3UTR/T). The C-allele 3′ UTR-containing vector (pLuc-3UTR/C) was obtained by a site-directed mutation using the QuikChange II XL mutagenesis kit (Stratagene, La Jolla, CA, USA) and the following designed primers: 5′-gaggagattgctctggggcttgctattaagaaactaag-3′ (forward) and 5′-cttagtttcttaatagcaagccccagagcaatctcctc-3′ (reverse). All constructs were confirmed by DNA sequencing.

The human neuroblastoma SH-SY5Y cell line was purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured as suggested by the vendor. The transfections were carried out with Lipofectamine 2000 from Invitrogen (Carlsbad, CA, USA), according to the manufacturer's protocol. Luciferase activities in cells after 48 h of transfection were analyzed using the Luciferase Assay System with the $20/20^n$ luminometer method (Promega). The two allele constructs, pLuc-3UTR/C and pLuc-3UTR/T, were compared with pLuc as a control. For each plasmid construction, quadruplicate transfections were performed. Their luciferase activities were assayed separately and then averaged in each experiment. Three independent experiments were conducted for replication.

Table 2 Minor allele frequencies of polymorphisms in AA, EA, and pooled samples

SNP no.	dbSNP ID	Allele[a]	AA	EA	Pooled
1	rs265973	C/T	0.41	0.45	0.42
2	rs265975	T/C	0.37	0.38 (T)	0.44
3	rs686	A/G	0.42	0.36 (A)	0.48
4	rs4532	T/C	0.12	0.35	0.18
5	rs2168631	A/G	0.25	0.18	0.23

[a] Second allele is listed as minor allele unless noted in parentheses

Results

rs686 and rs4532 in *DRD1* are significantly associated with ND

We genotyped five SNPs, two within and three near *DRD1*. The tests for HWE indicated that no SNP deviated significantly from HWE in either the AA or EA sample (minimum $P = 0.17$ and 0.50 for the AA and EA samples, respectively), confirming the high quality of the genotyping data. The minor allele frequency of each SNP in the AA, EA, and pooled subjects is shown in Table 2. In light of the ethnic-specific characteristics of SNPs among ethnic groups (Gabriel et al. 2002; Wall and Pritchard 2003) and the known ethnic differences in ND and nicotine metabolism (Benowitz et al. 1999; Perez-Stable et al. 1998), we performed separate association analysis on each ethnic sample as well as on the pooled sample.

Of five SNPs examined, rs265973 was significantly associated with SQ ($P = 0.041$); rs686 with SQ ($P = 0.0078$), HSI ($P = 0.0093$), and FTND ($P = 0.0048$); and rs4532 with FTND ($P = 0.035$) in the AA sample. However, only the association of rs686 with the three ND measures remained significant after correction for multiple testing on the basis of the SNPSpD approach (Nyholt 2004) (Table 3). In the EA sample, we found a significant association only of rs4532 with FTND prior to correction for multiple testing ($P = 0.035$). In the pooled sample, we found significant associations of rs265975, rs686, and rs4532 with all three ND measures, and the associations of rs686 and rs4532 with HSI and FTND remained significant even after correction for multiple testing (Table 3).

Further, we performed haplotype-based association analysis for these five SNPs. We first evaluated pair-wise LD among them with Haploview (Barrett et al. 2005) to define haplotype blocks according to the criteria specified by Gabriel et al. (2002). Unexpectedly, we detected no haplotype block in LD structures from our AA, EA, and pooled samples, even though some of these SNPs are only approximately 1.0 kb apart (Fig. 1). We then employed the FBAT program (Horvath et al. 2004) to assess ND association with all three contiguous SNPs using a sliding window approach (Lin et al. 2004). As shown in Table 4, our haplotype-based association analysis revealed a haplotype, C-T-A, formed by rs265973–rs265975–rs686, that was significantly associated with all three ND measures in AAs (frequency = 21%; number of families = 148; $Z = -3.3$; $P < 0.001$) as well as in the pooled sample (frequency = 19%; number of families = 190; $Z = -3.0$; $P < 0.005$). All these associations remained significant after Bonferroni correction for all major haplotypes. In addition, we found that another haplotype, T-A-T, formed by rs265975–rs686–rs4532, yielded significant associations with all three ND measures in the pooled sample. However, only the association with FTND remained significant after Bonferroni correction for all major haplotypes (frequency = 32%; number of families = 274; $Z = -2.8$; $P = 0.0055$; Table 4).

rs686 alters reporter gene expression

The results from both PBAT and FBAT analyses implied that the rs686 polymorphism is a causative locus for the association of *DRD1* with ND. Sequence analysis indicated

Table 3 *P* values for association of individual SNPs with three ND measures in AA, EA, and pooled samples under additive model

SNP no.	dbSNP ID	AA			EA			Pooled		
		SQ	HSI	FTND	SQ	HSI	FTND	SQ	HSI	FTND
1	rs265973	0.041	0.059	0.056	0.78	0.93	0.89	0.065	0.10	0.13
2	rs265975	0.14	0.076	0.063	0.16	0.17	0.098	0.041	0.025	0.015
3	rs686	**0.0078**	**0.0093**	**0.0048**	0.77	0.50	0.35	0.017	**0.010**	**0.0040**
4	rs4532	0.080	0.073	0.035	0.16	0.068	0.035	0.025	**0.0097**	**0.0026**
5	rs2168631	0.78	0.94	0.77	0.28	0.37	0.34	0.44	0.62	0.81

Significant *P* values after correction for multiple testing are given in bold. The adjusted *P* value at the 0.05 significance level is 0.010 for AAs, 0.013 for EAs, and 0.011 for pooled sample. Age and sex were included as covariates for each ethnic-specific sample. Additional ethnicity was adjusted for pooled sample

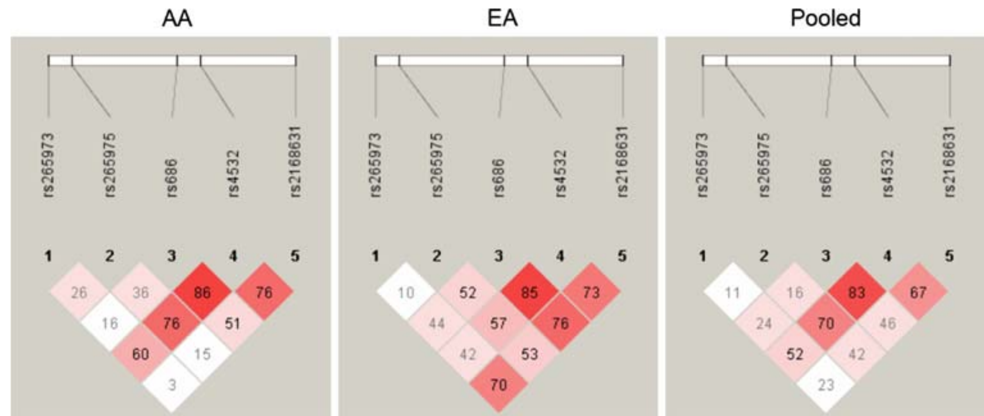

Fig. 1 Haploview-generated LD patterns of five SNPs within or near *DRD1* in AA, EA, and pooled samples. *Number* in each box represents D' value for each SNP pair

Table 4 *Z* scores and permutated *P* values for haplotype association of consecutive three SNPs with ND measures under additive model

Haplotype[a]					AA				EA				Pooled			
1	2	3	4	5	%[b]	SQ	HIS	FTND	%[b]	SQ	HSI	FTND	%[b]	SQ	HSI	FTND
C	T	A			21	**−3.4**	**−3.3**	**−3.3**	16	−0.034	−0.019	−0.024	19	**−3.1**	**−2.9**	**−2.9**
						0.00059	**0.00094**	**0.00086**		0.97	0.98	0.98		**0.0021**	**0.0036**	**0.0039**
	T	A	T		32	−1.8	−1.6	−1.8	31	−1.5	−2.0	−2.2	32	−2.4	−2.5	**−2.8**
						0.066	0.11	0.070		0.12	0.049	0.025		0.015	0.014	**0.0055**
		A	T	A	16	−1.9	−1.9	−1.7	17	−0.8	−0.9	−0.9	16	−2.0	−2.0	−1.9
						0.060	0.061	0.081		0.41	0.39	0.37		0.050	0.049	0.062

Significant *Z* scores and permuted *P* values are shown in bold. Adjusted permutation *P* value at the 0.05 significance level after Bonferroni correction for five major haplotypes is 0.010. Age and sex were included as covariates for each ethnic-specific sample. Additional ethnicity was adjusted for pooled sample

[a] SNP no. instead of dbSNP ID is used here

[b] Haplotype frequency

that rs686 is located in the 3′ UTR and that the regional sequence around rs686 is conserved among human, mouse, and rat (Fig. 2a). Because the 3′ UTR has been recognized as a regulatory element of messenger RNA (mRNA) for post-transcriptional gene regulation, we used a luciferase reporter assay to investigate whether rs686 variant causes differential gene expression.

We cloned the full-length 3′ UTR of *DRD1* and fused it with the firefly luciferase gene to form a chimeric reporter gene (Fig. 2a). The reporter assay in human neuroblastoma SH-SY5Y cells demonstrated that the chimeric reporter gene bearing a *DRD1* 3′ UTR with the rs686/G allele was associated with a significant lower luciferase activity (~27%; $P < 0.05$) than the one containing the rs686/A allele; and that both chimeric reporter genes yielded significantly lower luciferase activities (~50%; $P < 0.01$) than the control without the *DRD1* 3′ UTR (Fig. 2b). This indicates that the 3′ UTR is functionally involved in regulation of *DRD1* expression, and that *DRD1* with the rs686/A allele has a higher expression level than does *DRD1* with the rs686/G allele.

Discussion

In this study, we first demonstrated that rs686 in *DRD1* is significantly associated with ND in AAs, whereas rs686 and rs4532 are significantly associated with ND in the pooled sample. We then showed that haplotype C-T-A, formed by rs265973–rs265975–rs686, is significantly associated with ND in both AA and pooled samples, and that another haplotype, T-A-T, formed by rs265975–rs686–rs4532, is significantly associated with FTND in the pooled sample. Finally, we provided evidence that rs686 variant causes a differential expression of a chimeric reporter gene bearing the *DRD1* 3′ UTR, suggesting that rs686 is a functional polymorphism affecting dopamine D_1 receptor expression.

The D_1 receptor is one of the major receptors in the brain that mediate the actions of the neurotransmitter dopamine in a variety of psychomotor functions. Because of the suggestive evidence of dopaminergic system dysfunction and its role in the pathogenesis of neuropsychiatric disorders, *DRD1* has been one of the genes investigated most extensively

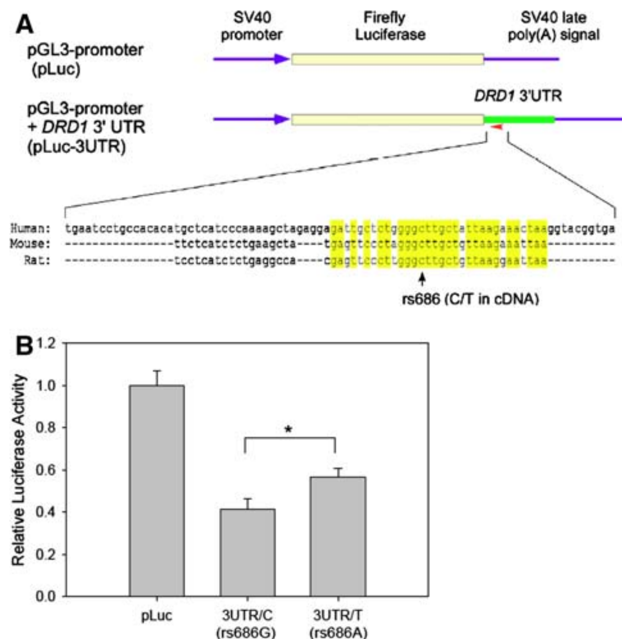

Fig. 2 rs686 affects expression of chimeric luciferase gene bearing *DRD1* 3′ UTR. **a** Plasmid constructs for luciferase reporter assay. The 3′ UTR of *DRD1* with rs686 G or A allele was cloned to fuse with firefly luciferase reporter gene (in pLuc) to form chimeric gene (in pLuc-3UTR/C or pLuc-3UTR/T). Sequence around rs686 in 3′ UTR of human *DRD1* was aligned with its counterparts from mouse and rat, which demonstrates that rs686 is located in conserved region. The A/G transition of rs686 is shown as C/T transition in cDNA sequence, so-called *Bsp* 1286I (T1403C) polymorphism. **b** Luciferase activities in vector-transfected cells. Chimeric reporter gene with *DRD1* 3′ UTR in pLuc-3UTR/C (rs686G) or pLuc-3UTR/T (rs686A) vector was compared with control reporter gene without *DRD1* 3′ UTR in pLuc vector. Luciferase activities in human neuroblastoma SH-SY5Y cells were measured after 48 h of cell transfection. Result is representative of three independent experiments. Data shown as mean ± SD ($N = 4$). *: $P < 0.05$, paired Student's *t*-test

in these diseases. Recently, molecular genetic analysis revealed that *DRD1* is associated with bipolar disorder (Del Zompo et al. 2007; Severino et al. 2005), ADHD (Bobb et al. 2005; Misener et al. 2004), ND (Comings et al. 1997), and alcohol dependence (Kim et al. 2007; Limosin et al. 2003). The *Dde*I (A-48G) polymorphism in the 5′ UTR (rs4532) has been reported to be significantly associated with compulsive, addictive behaviors (Comings et al. 1997; Kim et al. 2007; Limosin et al. 2003). In contrast, the *Bsp*1286I (T1403C) polymorphism in the 3′ UTR of *DRD1* (rs686) has not been associated with alcoholism (Sander et al. 1995) or ND.

For the first time, we demonstrated here that both the rs4532 and the rs686 polymorphisms are significantly associated with ND. For rs4532, the significant association with FTND was shown in our ethnic-specific and pooled samples. However, the significance levels in the AA and EA samples were much weaker than that in the pooled sample, probably because of the larger size of the pooled sample. With a larger size and greater statistical power in the pooled sample, we were able to detect a more significant association of rs4532 with ND. For rs686, significant associations with all three ND measures were found in both the AA and the pooled samples, but not in the EA sample. In the haplotype-based analysis, we also revealed significant association of haplotype C-T-A, formed by rs265973–rs265975–rs686, with all three ND measures in the AA and pooled samples, but not in the EA sample. This is largely attributable to the different ethnic-specific characteristics of rs265975 and rs686 (see their minor allele frequency differences in Table 2). Of interest, the *Z* scores of all three haplotypes in Table 4 were revealed to be negative, indicating that these haplotypes are protective against ND.

Both the rs4532 and the rs686 polymorphisms have base changes outside the coding region of *DRD1*. Because of their unknown functions, their associations, if real, might reflect LD with other polymorphisms in regions at or near a locus that affects *DRD1* function or density (Comings et al. 1997). However, to date, no polymorphisms have been found to change the amino acid sequence in the *DRD1* coding region, and no polymorphisms of the 5′ flanking regulatory region of *DRD1* have been revealed to have an impact on the transcriptional activity (Cichon et al. 1996). Thus, we suspect that polymorphisms located within the 5′ UTR and 3′ UTR of *DRD1* are likely to affect *DRD1* expression. By using an in vitro reporter assay, we demonstrated that the allele variants of polymorphism rs686, located in 3′ UTR of *DRD1*, produced different expression levels of the chimeric luciferase gene, implying that rs686 is functional in *DRD1* expression. Besides, It has been reported that the 5′ UTR of eukaryotic cellular mRNA can cross-talk with its 3′ UTR in protein translation to influence gene expression (Komarova et al. 2006). Located in the 5′ UTR of *DRD1*, the rs4532 polymorphism may also affect *DRD1* expression. New evidence that the rs4532 polymorphism is of functional importance came from a preliminary pharmacogenetics study. On positron emission tomography scans, Potkin et al. (2003) observed that brain regional metabolic and clinical responses to clozapine in treatment-resistant schizophrenic subjects were significantly related to the genotype at the *Dde* I polymorphism site.

Polymorphism rs686 had a subtle influence on gene expression in our in vitro reporter assay. Such an influence is possibly caused by either direct or indirect microRNA (miRNAs) targeting, an emerging class of regulatory genes with about 20–25 nucleotides (Lewis et al. 2005), could regulate mRNAs homeostasis in the cell. It has been estimated that the 3′ UTRs of more than a third of coding genes are targeted by miRNAs (Bartel 2004). The binding of miRNAs to an mRNA 3′ UTR can initiate mRNA degradation or translational repression, which post-transcriptionally regulates gene expression (Bartel 2004).

Polymorphism rs686 may cause differential stability of *DRD1* mRNA and thus affect *DRD1* expression. One possibility is that rs686 resides right on the target site of an miRNA, which directly affects the miRNA binding to *DRD1* 3′ UTR. Another possibility is that rs686 changes *DRD1* mRNA secondary structure, which indirectly affects miRNA(s) binding to the *DRD1* 3′ UTR. Nonetheless, further experiments are needed to prove such a speculation above.

Our study demonstrates that *DRD1* is significantly associated with ND from both single SNP- and haplotype-based analysis, and that polymorphisms rs686 and rs4532 represent two important SNP loci of the gene. We reveal that the rs686 polymorphism is functional and propose that the rs4532 polymorphism is functional as well. Both polymorphisms may affect the *DRD1* expression level and the density of the dopamine D_1 receptor, and probably are causative SNPs for ND. Because rs4532 resides in the *DRD1* 5′ UTR and rs686 in the 3′ UTR, it will be of great interest to investigate their synergistic effects in *DRD1* expression and in pharmacogenetic association with ND. Future studies are required to uncover the relationships among these two *DRD1* polymorphisms, *DRD1* expression levels and dopamine D_1 receptor densities, and ND, which we hope will provide significant insight into the pathophysiological mechanisms of ND.

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References

Barrett JC, Fry B, Maller J, Daly MJ (2005) Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 21:263–265

Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116:281–297

Benowitz NL, Perez-Stable EJ, Fong I, Modin G, Herrera B, Jacob P 3rd (1999) Ethnic differences in N-glucuronidation of nicotine and cotinine. J Pharmacol Exp Ther 291:1196–1203

Beuten J, Payne TJ, Ma JZ, Li MD (2006) Significant association of catechol-O-methyltransferase (COMT) haplotypes with nicotine dependence in male and female smokers of two ethnic populations. Neuropsychopharmacology 31:675–684

Beuten J, Ma JZ, Payne TJ, Dupont RT, Lou XY, Crews KM, Elston RC, Li MD (2007) Association of specific haplotypes of neurotrophic tyrosine kinase receptor 2 gene (NTRK2) with vulnerability to nicotine dependence in African-Americans and European-Americans. Biol Psychiatry 61:48–55

Bobb AJ, Addington AM, Sidransky E, Gornick MC, Lerch JP, Greenstein DK, Clasen LS, Sharp WS, Inoff-Germain G, Wavrant-De Vrieze F, Arcos-Burgos M, Straub RE, Hardy JA, Castellanos FX, Rapoport JL (2005) Support for association between ADHD and two candidate genes: NET1 and DRD1. Am J Med Genet B Neuropsychiatr Genet 134:67–72

Cichon S, Nothen MM, Erdmann J, Propping P (1994) Detection of four polymorphic sites in the human dopamine D1 receptor gene (DRD1). Hum Mol Genet 3:209

Cichon S, Nothen MM, Stober G, Schroers R, Albus M, Maier W, Rietschel M, Korner J, Weigelt B, Franzek E, Wildenauer D, Fimmers R, Propping P (1996) Systematic screening for mutations in the 5′-regulatory region of the human dopamine D1 receptor (DRD1) gene in patients with schizophrenia and bipolar affective disorder. Am J Med Genet 67:424–428

Comings DE, Gade R, Wu S, Chiu C, Dietz G, Muhleman D, Saucier G, Ferry L, Rosenthal RJ, Lesieur HR, Rugle LJ, MacMurray P (1997) Studies of the potential role of the dopamine D1 receptor gene in addictive behaviors. Mol Psychiatry 2:44–56

Del Zompo M, De Luca V, Severino G, Ni X, Mulas S, Congiu D, Piccardi MP, Kennedy JL (2007) Haplotype association study between DRD1 gene and bipolar type I affective disorder in two samples from Canada and Sardinia. Am J Med Genet B Neuropsychiatr Genet 144:237–241

Duggirala R, Almasy L, Blangero J (1999) Smoking behavior is under the influence of a major quantitative trait locus on human chromosome 5q. Genet Epidemiol 17(Suppl 1):S139–S144

Gabriel SB, Schaffner SF, Nguyen H, Moore JM, Roy J, Blumenstiel B, Higgins J, DeFelice M, Lochner A, Faggart M, Liu-Cordero SN, Rotimi C, Adeyemo A, Cooper R, Ward R, Lander ES, Daly MJ, Altshuler D (2002) The structure of haplotype blocks in the human genome. Science 296:2225–2229

Heatherton TF, Kozlowski LT, Frecker RC, Fagerstrom KO (1991) The Fagerstrom Test for nicotine dependence: a revision of the Fagerstrom Tolerance Questionnaire. Br J Addict 86:1119–1127

Ho MK, Tyndale RF (2007) Overview of the pharmacogenomics of cigarette smoking. Pharmacogenomics J 7:81–98

Horvath S, Xu X, Lake SL, Silverman EK, Weiss ST, Laird NM (2004) Family-based tests for associating haplotypes with general phenotype data: application to asthma genetics. Genet Epidemiol 26:61–69

Kim DJ, Park BL, Yoon S, Lee HK, Joe KH, Cheon YH, Gwon DH, Cho SN, Lee HW, NamGung S, Shin HD (2007) 5′ UTR polymorphism of dopamine receptor D1 (DRD1) associated with severity and temperament of alcoholism. Biochem Biophys Res Commun 357:1135–1141

Komarova AV, Brocard M, Kean KM (2006) The case for mRNA 5′ and 3′ end cross talk during translation in a eukaryotic cell. Prog Nucleic Acid Res Mol Biol 81:331–367

Lange C, DeMeo D, Silverman EK, Weiss ST, Laird NM (2004) PBAT: tools for family-based association studies. Am J Hum Genet 74:367–369

Lewis BP, Burge CB, Bartel DP (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120:15–20

Li MD (2006) The genetics of nicotine dependence. Curr Psychiatry Rep 8:158–164

Li MD, Cheng R, Ma JZ, Swan GE (2003) A meta-analysis of estimated genetic and environmental effects on smoking behavior in male and female adult twins. Addiction 98:23–31

Li MD, Ma JZ, Beuten J (2004) Progress in searching for susceptibility loci and genes for smoking-related behaviour. Clin Genet 66:382–392

Li MD, Beuten J, Ma JZ, Payne TJ, Lou XY, Garcia V, Duenes AS, Crews KM, Elston RC (2005) Ethnic- and gender-specific association of the nicotinic acetylcholine receptor alpha4 subunit gene (CHRNA4) with nicotine dependence. Hum Mol Genet 14:1211–1219

Li MD, Sun D, Lou XY, Beuten J, Payne TJ, Ma JZ (2007) Linkage and association studies in African- and Caucasian-American

populations demonstrate that SHC3 is a novel susceptibility locus for nicotine dependence. Mol Psychiatry 12:462–473

Limosin F, Loze JY, Rouillon F, Ades J, Gorwood P (2003) Association between dopamine receptor D1 gene DdeI polymorphism and sensation seeking in alcohol-dependent men. Alcohol Clin Exp Res 27:1226–1228

Lin S, Chakravarti A, Cutler DJ (2004) Exhaustive allelic transmission disequilibrium tests as a new approach to genome-wide association studies. Nat Genet 36:1181–1188

Misener VL, Luca P, Azeke O, Crosbie J, Waldman I, Tannock R, Roberts W, Malone M, Schachar R, Ickowicz A, Kennedy JL, Barr CL (2004) Linkage of the dopamine receptor D1 gene to attention-deficit/hyperactivity disorder. Mol Psychiatry 9:500–509

Nisell M, Nomikos GG, Svensson TH (1994) Systemic nicotine-induced dopamine release in the rat nucleus accumbens is regulated by nicotinic receptors in the ventral tegmental area. Synapse 16:36–44

Nyholt DR (2004) A simple correction for multiple testing for single-nucleotide polymorphisms in linkage disequilibrium with each other. Am J Hum Genet 74:765–769

O'Connell JR, Weeks DE (1999) An optimal algorithm for automatic genotype elimination. Am J Hum Genet 65:1733–1740

Perez-Stable EJ, Herrera B, Jacob P 3rd, Benowitz NL (1998) Nicotine metabolism and intake in black and white smokers. JAMA 280:152–156

Pontieri FE, Tanda G, Orzi F, Di Chiara G (1996) Effects of nicotine on the nucleus accumbens and similarity to those of addictive drugs. Nature 382:255–257

Potkin SG, Basile VS, Jin Y, Masellis M, Badri F, Keator D, Wu JC, Alva G, Carreon DT, Bunney WE Jr, Fallon JH, Kennedy JL (2003) D1 receptor alleles predict PET metabolic correlates of clinical response to clozapine. Mol Psychiatry 8:109–113

Saccone NL, Neuman RJ, Saccone SF, Rice JP (2003) Genetic analysis of maximum cigarette-use phenotypes. BMC Genet 4(Suppl 1):S105

Sander T, Harms H, Podschus J, Finckh U, Nickel B, Rolfs A, Rommelspacher H, Schmidt LG (1995) Dopamine D1, D2 and D3 receptor genes in alcohol dependence. Psychiatr Genet 5:171–176

Severino G, Congiu D, Serreli C, De Lisa R, Chillotti C, Del Zompo M, Piccardi MP (2005) A48G polymorphism in the D1 receptor genes associated with bipolar I disorder. Am J Med Genet B Neuropsychiatr Genet 134:37–38

Sullivan PF, Kendler KS (1999) The genetic epidemiology of smoking. Nicotine Tob Res 1(Suppl 2):S51–S57 (discussion S69–S70)

Vink JM, Posthuma D, Neale MC, Eline Slagboom P, Boomsma DI (2006) Genome-wide linkage scan to identify loci for age at first cigarette in Dutch sibling pairs. Behav Genet 36:100–111

Wall JD, Pritchard JK (2003) Haplotype blocks and linkage disequilibrium in the human genome. Nat Rev Genet 4:587–597

Wong AH, Buckle CE, Van Tol HH (2000) Polymorphisms in dopamine receptors: what do they tell us? Eur J Pharmacol 410:183–203

World Health Organization Tobacco Free Initiative (2005) Waterpipe tobacco smoking: health effects, research needs and recommended actions by regulators. World Health Organization, Geneva